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Amplification of fluorescent in situ hybridisation signals in formalin fixed paraffin wax embedded sections of colon tumour using biotinylated tyramide.
McKay J A; Murray G I; Keith W N; McLeod H L
Department of Medicine and Therapeutics, University of Aberdeen, Institute of Medical Sciences, Foresterhill, UK. j.a.mckay@abdn.ac.uk
Molecular pathology - MP (ENGLAND) Dec 1997, 50 (6) p322-5, ISSN 1366-8714 Journal Code: 9706282

2) **A new technique for cyclic in situ amplification and a case report about amplification of a single** copy gene **sequence in human metaphase chromosomes through PCR-PRINS.**
Harrer T; Schwinger E; Mennicke K
Department of Human Genetics, Medical University of Lubeck, Lubeck, Germany. mennicke@medinf.mu-luebeck.de
Human mutation (United States) Feb 2001, 17 (2) p131-40, ISSN 1098-1004 Journal Code: 9215429

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HUMAN MUTATION 17:131-140 (2001)

METHODS

A New Technique for Cyclic In Situ Amplification and a Case Report About Amplification of a Single Copy Gene Sequence in Human Metaphase Chromosomes Through PCR-PRINS

Thomas Harrer,[1] Eberhard Schwinger,[1] and Kirsten Mennicke[1,2]*
[1]*Department of Human Genetics, Medical University of Lübeck, Lübeck, Germany*
[2]*Klinik für Kinder- und Jugendmedizin, Medizinische Universität zu Lübeck, Lübeck, Germany*

Communicated by Martin Bobrow

Since the introduction of PRimed IN Situ labeling (PRINS) as a rapid and extremely sensitive alternative method to conventional fluorescence in situ hybridization (FISH), its application in clinical cytogenetics has been limited to the detection of highly repeated sequences, such as centromeric and telomeric regions. In the original PRINS method, unlabeled oligonucleotide probes are annealed to their repeated complementary target sequences in fixed human metaphase chromosomes on a slide. The probes serve as primers for subsequent in situ chain elongation with Taq DNA polymerase and labeled nucleotides. In contrast to conventional PCR, cyclic in situ amplification of the chromosomal target DNA with paired primers remained both difficult and strictly limited to highly repeated sequences, since the maintenance of constant reaction conditions on the slide during temperature and pressure shifts presents a major problem. We developed a new system for in situ PCR that allows the amplification of target sequences analogous to PCR in the test tube. We applied this method successfully for the detection of highly repeated sequences, for the detection of low copy repeats, and in one case, for the detection of a single-copy DNA sequence. The significance of this development for further in situ PCR applications will be discussed. Hum Mutat 17:131–140, 2001.

KEY WORDS: primed in situ labeling; PRINS; cyclic PRINS; in situ PCR; FISH; single-copy gene sequence; single-copy gene amplification; human metaphase chromosomes; DMD; APOA1

DATABASES:
DMD – OMIM: 310200; GDB:119850; HGMD: DMD
APOA1 – OMIM: 107680; GDB:119684; HGMD: APOA1

INTRODUCTION

The PRimed IN Situ labeling (PRINS) method is based upon the sequence-specific hybridization of unlabeled oligonucleotides to their complementary target sequences in the chromatin where they serve as primers for a chain elongation reaction in situ catalyzed by a DNA polymerase [Koch et al., 1989]. The binding sites are visualized directly or indirectly through incorporation of either fluorochrome-labeled or haptenized nucleotides into the newly synthesized DNA strand. Haptenized binding sites are subsequently detected

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*Correspondence to: Dr. med. Kirsten Mennicke, Klinik für Kinder- und Jugendmedizin, Medizinische Universität zu Lübeck, Ratzeburger Allee 160, D-23538 Lübeck, Germany. E-mail: mennicke@medinf.mu-luebeck.de

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by fluorochrome-labeled antibodies. The use of oligonucleotide probes allows the detection of much smaller targets than those accessible to conventional in situ hybridization and results in the higher sensitivity of the PRINS method and in its amazing discriminatory power for small sequence variations [Pellestor et al., 1994, 1995].

Beyond the fast characterization of both numerical and structural chromosome aberrations, it is likely that modifications of the PRINS method will provide a bigger field of future applications in the detection of low- and single-copy DNA sequences. Possible routine detection of single copy genes would allow the physical mapping of short sequences from partially sequenced DNA (e.g., sequence tagged sites and expressed sequence tags) to human chromosomes as well as detecting the deletion of a single exon by cytogenetic analysis. As opposed to FISH, the PRINS method provides the possibility for the enhancement of its sensitivity by performing multiple cycles of PRINS reactions [Gosden and Hanratty, 1993; Therkelsen et al., 1993; Gosden and Lawson, 1994]. While the exponential in situ amplification of highly repeated sequences has been accomplished with paired primers [Therkelsen et al., 1993], this has not been possible for single-copy genes. With our new cycling PRINS technique we present here the first human single-copy gene amplification. We amplified a sequence of 273 bp in exon 8 of the DMD gene (MIM# 310200).

MATERIALS AND METHODS

Chromosomes were prepared from peripheral blood lymphocytes by standard methods, fixed in methanol: acetic acid (3:1) and dropped onto clean glass slides above a center-cross previously carved on the bottom side of the slide. Then the slides were air-dried and passed through an ethanol series (70%, 90%, 100%), were air-dried again and aged for three d at room temperature. We used a ddNTP pretreatment [Koch et al., 1995] in order to avoid non-specific initiation of chain elongation on single-strand nicks in the chromosomal DNA [Gosden and Lawson, 1994]. The reaction mixture for the ddNTP pretreatment was applied in a "slide seal" reaction chamber (Hybaid Corp., Teddington, UK) and consisted of 40µl distilled water, 5µl of 10×Taq-buffer (500 mM KCl, 15 mM $MgCl_2$, 100 mM TrisHCl pH 8.3), 5µl of 1mM ddNTP-sol. and 0.3µl Taq-polymerase (5U/µl) (Oncor, Appligene No. 120181A). The slides were incubated at 65°C for 20 min and then were transferred to stop buffer (50 mM NaCl, 50 mM EDTA, pH 8) for 1 min at room temperature, followed by two washing cycles of 5 min each in 2×SSC. The slides were then passed through an ethanol wash series (70%, 90%, 100%, 3 min each), air-dried again, and incubated in 70% deionized formamide in 2×SSC at 70°C for 2 min to denature the chromosomal DNA for the first PRINS cycle. This procedure was immediately followed by an ice-cold ethanol series (70%, 90%, 100%, 3 min each) at –20°C. After being air-dried again the slides can be stored in a folder at –20°C up to six weeks.

The key trick for our multicyclic PRINS protocol is the use of an open reaction chamber for a reaction volume of 75µl. Evaporation control is provided by an additional layer of 70µl paraffin oil over the reaction mixture. As reaction chambers we took the upper part of PCR-tubes (No. 710910; Biozym Diagnostik Corp., Olgendorf, Germany). A hot blade enabled us to cut them in large numbers. With a hot glue pistol (Pattex Supermatic, www.henkel.com) we applied silicon glue (Pattex hot sticks transparent; Henkel) in the shape of a ring on the slide and then set the cut tube piece into the ring with the cut side facing upward. The application of the silicon glue onto the slide is facilitated by placing the slide on a paper with the drawing of a circle that corresponds to the diameter of the PCR-tube, the carved center-mark of the slide being adjusted to the center of the drawing. For our reaction mix we used deep frozen aliquots of 60µl containing 1.5µl of each 10mM dATP, dCTP, dGTP; 1.0µl of 10mM dTTP; 4µl of glycerol 80%; 7.5µl of 10×Taq-buffer and 43µl of distilled water. To the aliquot were added before incubation: 5µl of 1mM digoxigenin-11-dUTP, oligonucleotide primer solution (Table 1) and distilled water to the total volume of 75µl. The nucleotide concentrations in the volume of 75µl are:

$$[dATP] = [dCTP] = [dGTP] = [dTTP] + [dig\text{-}dUTP] = 0.2\ mM.$$

TABLE 1. Sequences of Oligonucleotide Primers for Cyclic PRINS and PCR-PRINS

Name		Origin	Sequence
Primers for highly repeated sequences:			
DXZ1-F	(23-mer)	Warburton et al. [1991]	ATA ATT TCC CAT AAC TAA ACA CA
DXZ1-R	(23-mer)	Warburton et al. [1991]	TGT GAA GAT AAA GGA AAA GGC TT
Amplicon of DXZ1-F+R: ~500bp		best annealing temp.: 60°–64°C (titrated)	
All telomeres	(42-mer)	Therkelsen et al. [1995]	$(CCC\ TAA)_7$
			best annealing temp.: 60°–62°C (titrated)
1p36	(30-mer)	Koch et al. [1995]	STG GGT GCT GTT CCA GGC TGT CAG AGG CTC
			best annealing temp.: 60°–65°C (titrated)
Primers for low repetitive sequences on the example of the apo (a)-gene:			
Apo F	(30-mer)	Hindkjaer et al. [1996]	GGG TGC AGG AGT GCT ACC ATG GTA ATG GAC
Apo R	(24-mer)	Hindkjaer et al. [1996]	CCC AGG CCT TTG CTC AGT CGG GTG
			best annealing temp.: 64°–66°C (titrated)
Apo-2 F	(30-mer)	Mennicke and Harrer	TCA TGA ACT ACT GCA GGA ATC CAG ATG CTG
Apo-2 R	(28-mer)	Mennicke and Harrer	GTT CGG AAG GAG CCT CTA GGC TTG GGA A
Amplicon of Apo-2F+R: 172 bp			best annealing temp.: 62°–64°C (titrated)
Primers for single copy DNA-sequences on the the example of the DMD-gene:			
Primer mix of the smallest amplicons:			
DMD-Ex53-F	(27-mer)	Abbs et al. [1991]	TTG AAA GAA TTC AGA ATC AGT GGG ATG
DMD-Ex53-R	(29-mer)	Abbs et al. [1991]	CTT GGT TTC TGT GAT TTT CTT TTG GAT TG
Amplicon: 212 bp			theoret. optimal annealing temp.: 54.8°C
DMD-Ex60-F	(29-mer)	Beggs et al. [1990]	AGG AGA AAT TGC GCC TGT GAA AGA GAA CG
DMD-Ex60-R	(28-mer)	Beggs et al. [1990]	CTG CAG AAG CTT CCA TCT GGT GTT CAG G
Amplicon: 139 bp			theoret. optimal annealing temp.: 58.2°C
DMD-Ex8-iiF	(29-mer)	Mennicke and Harrer	TCT GGA GGA CAT TCA TGG ACA ATT CAC TG
DMD-Ex8-iiR	(29-mer)	Mennicke and Harrer	TTC TTT AGT CAC TTT AGG TGG CCT TGG CA
Amplicon: 273 bp			theoret. optimal annealing temp.: 54.9°C
			best annealing temp.: 64° (titrated)
DMD-Ex44-iiF	(25-mer)	Mennicke and Harrer	ACA GAT CTG TTG AGA AAT GGC GGC G
DMD-Ex44-iiR	(30-mer)	Mennicke and Harrer	TCC CAA TTC TCA GGA ATT TGT GTC TTT CTG
Amplicon: 112 bp			theoret. optimal annealing temp.: 52.2°C
DMD-Ex51-iiF	(31-mer)	Mennicke and Harrer	ATT TTT CTT TTT CTT CTT TTT TCC TTT TTG C
DMD-Ex51-iiR	(30-mer)	Mennicke and Harrer	TCA TAC CTT CTG CTT GAT GAT CAT CTC GTT
Amplicon: 290 bp			theoret. optimal annealing temp.: 55.1°C
DMD-Ex53-iiF	(26-mer)	Mennicke and Harrer	AAG AAC ACC TTC AGA ACC GGA GGC AA
DMD-Ex53-iiR	(29-mer)	Mennicke and Harrer	TGT ATA GGG ACC CTC CTT CCA TGA CTC AA
Amplicon: 143 bp			theoret. optimal annealing temp.: 56.3°C
DMD-Ex60-iiF	(29-mer)	Mennicke and Harrer	TGA GCC ACG TCA AGG ACC TTG CTC GCC AG
DMD-Ex60-iiR	(31-mer)	Mennicke and Harrer	TGA GGT TAT ACG GTG AGA GCT GAA TGC CCA A
Amplikon: 69 dp			theoret. optimal annealing temp.: 55.9°C

The relation of the labeled nucleotides is:

$$[dTTP]:[dig\text{-}dUTP] = 2:1.$$

We used varying amounts of primers from 0.5µg (DMD-gene) up to 16µg apo(a)-gene (APOA1; MIM# 107680) for each single primer per reaction. After filling the reaction mix into the reaction chamber and adding 70µl of paraffin oil, the slides were transferred to a HYBAID OmniGene flatbed thermal cycler and preheated to the primer-specific annealing temperature. Then 0.4µl Taq-polymerase (5U/µl) were added analogous to the hot start in PCR technique.

The program used for multicyclic PRINS reactions was: X°C, 5 min; 72°C, 10 min; (1st cycle); 94°C, 4 min; X°C, 3 min; 72°C, 6 min; (2nd cycle); 94°C, 1 min; X°C, 1 min; 72°C, 3 min; (3rd to 20th cycles). X°C is the primer-specific annealing temperature. Best results were obtained with 15 to 20 cycles. After the last extension the slides were held at 72°C. Pieces of plastic foil (10cm × 10cm) with a small slit were slipped over the reaction chamber of each slide. Then the reaction chambers were washed out with 1ml stop buffer. The reaction chamber and the plastic foil were quickly removed and each slide transferred for 1 min into a Coplin jar with stop buffer preheated to the primer-specific annealing temperature. Then the slides were passed through an ethanol series (70%, 90%, 100%, 3

min each), afterwards through xylol for 3 min, then they were passed again through an ethanol series (100%, 90%, 70%, 3 min each) and were washed in washing buffer (4×SSC, pH 7.0; 0.05 % Tween®) for at least 2 × 15 min. Then the slides were incubated with a solution of 5µl anti-digoxigenin-fluorescein Fab-fragments (Boehringer Mannheim, No 1207741) in 1ml of 1% blocking reagent (Boehringer Mannheim, No 1363514) for 10–30 min in the dark and were washed again in washing buffer 3 × for 5 min. Without allowing the slides to dry they were mounted with propidium iodide in antifade (Oncor Appligene, Gaithersburg, MD).

Besides the cycling PRINS protocol, we carried out two step reactions: PCR-PRINS, consisting of amplification cycles with unlabeled nucleotides, followed by cycles with labeled nucleotides and nested PCRs with subsequently used sets of different primer-pairs. For PCR-PRINS (Fig. 1) we used a reaction mixture with dTTP instead of digoxigenin-11-dUTP. The first 12 to 15 cycles were performed according to the multicyclic PRINS-protocol. While holding the slides on 72°C after the last extension

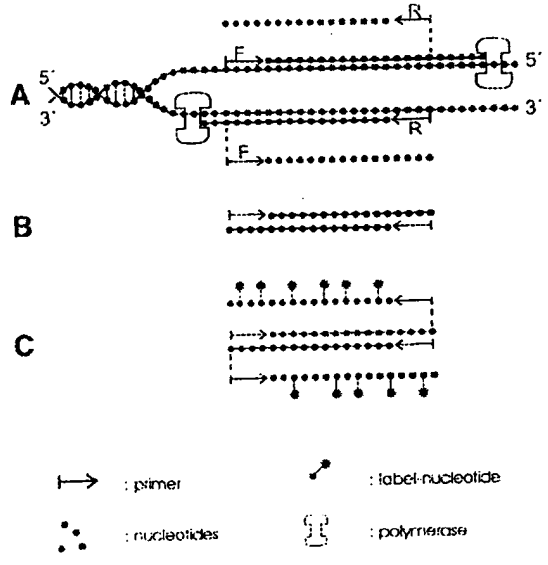

FIGURE 1. **Principles of PCR-PRINS. A: PCR in situ: 1st and 2nd cycle; B: PCR in situ: product after the 3rd cycle; C: Cycling PRINS.**

stage, 5µl of 1mM digoxigenin-11-dUTP and 1.5U Taq polymerase were added. Then the "PRINS-detection" was started: 94°C, 2 min; X°C, 3 min; 72°C, 6 min; (1st cycle); 94°C, 1 min; X°C, 1 min; 72°C, 3 min; (2nd to 5th cycle). The subsequent procedure was the same as for the multicyclic PRINS-protocol. Digital images were captured using a Hamamatsu charged-coupled device (CCD) camera mounted on a Zeiss Axiophot fluorescence microscope and were stored using Meta Systems software. Only hybridization with a hybridization rate over 80% after evaluating 50 metaphase spreads are reported.

Our protocols were carried out with single primers and primer-pairs (Table 1) that represent three groups of chromosomal targets: highly repeated sequences, low copy sequences, and single copy sequences. For highly repeated sequences we chose the midi-satellite sequence D1Z2 with its single primer "1p36" [Koch et al., 1995], centromeric α-satellite DNA with the primer pair DXZ1-F and DXZ1-R by Warburton et al. [1991] and the telomeric sequence with the 42-mer primer $(CCCTAA)_7$ by Therkelsen et al. [1995]. For low copy sequences we chose the apo(a) gene on chromosome 6q26-q27. This sequence contains a varying number (10–51) of similar or identical 342 bp Kringle IV motifs [Lackner et al., 1993]. We applied three different pairs of apo(a) primers: The primers published by Hindkjaer et al. [1996] are two independent single primers situated back to back: "Apo F" and "Apo R". We used the signal intensity obtained with "Apo F + Apo R" as our standard for linear amplification and expected stronger signal intensities for any exponential amplification within the apo(a) gene. We designed a primer pair "Apo-2 F+R" which produced specific amplicons of 172 bp on genomic DNA. The combination of the primer "Apo-2 F" with the primer "Apo R" resulted in a third primer pair and the production of a specific amplicon of 1385 bp. With this constellation we intended to compare the signal intensities of three primer-pairs in PRINS, which produced on genomic DNA: no amplicon, small amplicons, big amplicons.

For single copy sequences we chose the DMD

gene and constructed a set of primer-pairs with a primer-spacing of approximately 1 kb, according to the target size recommended for PRINS-labeling by Gosden and Lawson [1994]. We designed these primer-pairs as outer primers, nested around the well-established multiplex PCR-primer-pairs [Chamberlain et al., 1988, 1989; Beggs et al., 1990; Abbs et al., 1991]. The set of outer primer-pairs was incubated for several cycles with unlabeled nucleotides followed by a series of reaction cycles with the inner primer-pairs and labeled nucleotides. In a second approach we applied the same combined technique of nested PCR in situ and cyclic PRINS for a smaller primer-spacing: the original multiplex PCR-primer-pairs were used as outer primers and we designed a new set of primer-pairs nested inside their respective target sequences. In a third approach we selected seven primer-pairs with the smallest primer-spacing, ranging from 68 to 290 bp for a cyclic multiplex PRINS reaction with 12 amplification cycles. In a fourth approach we carried out both cyclic PRINS reactions and PCR-PRINS reactions (15 cycles unlabeled nucleotides / 5 cycles labeled nucleotides) for each one of the seven primer-pairs.

RESULTS

Application of Cycling PRINS to Highly Repeated Sequences

The primer pair DXZ1-F and DXZ1-R, originally designed for tube PCR on genomic DNA by Warburton et al. [1991], produced surprising results: Single PRINS reactions for each primer alone resulted in specific signals (Fig. 2A, B). With the primer pair we obtained stronger signals in a single reaction than with each primer alone (Fig. 2C). Our cycling PRINS-protocol with 20 cycles yielded no signal at all for each single primer (Fig. 2D), while the primer pair produced very strong specific signals in the cyclic protocol, with neither background noise nor signals on other centromeres (Fig. 2E). For the primer "1p36" [Koch et al., 1995] the situation is very different: Application of this single primer in a protocol of 20 cycles resulted in very strong signals (Fig. 3), stronger than the signals obtained in a single PRINS-reaction. Therkelsen et al. [1995] had applied the telomeric 42-mer primer $(CCCTAA)_7$ in a single-PRINS-protocol but did not receive signals on every telomere. When we applied the same primer in our cycling protocol we received strong and highly specific signals. After 20 cycles all telomeres were stained and no background or unspecific signals were obtained (Fig. 4).

Application of Our Cycling PRINS Protocol for the Detection of Low Copy Sequences

Each one of the four single apo(a) primers visualized their target sequence in a single-PRINS reaction (Fig. 5B). The specific signals were weak and associated with a lot of background staining. With primer pairs we received the best signals in our cycling PRINS protocol after 12 amplification cycles. All three primer-pairs showed specific signals of good quality and equal intensity (Fig. 5C–E). The best results for all apo(a) primer pairs were obtained with our two-step cycling protocol consisting of 15 cycles with unlabeled nucleotides, followed by five cycles with labeled nucleotides (Fig. 5F). However primer pair "Apo-2 F+R" produced still only marginally stronger signals than primers "Apo F + Apo R".

Application of Our Cycling PRINS Protocol for the Detection of Single Copy Sequences

The combination of multiplex-nested-PCR in situ and cycling PRINS revealed no specific signals for the DMD primer-sets: not for the nested primer pairs with big primer-spacing nor for the ones with small primer-spacing. The selection of seven primer pairs with the smallest primer-spacing (68 to 290 bp) applied as a multiplex primer cocktail revealed specific signals at the DMD-locus on chromosome Xp21 after a cyclic PRINS protocol with 12 amplification cycles. Subsequently we carried out cyclic PRINS reactions and PCR-PRINS reactions for each one of the seven primer pairs alone: Only one primer pair produced unambiguous specific signals (Fig. 6A–C). The PCR-PRINS protocol improved both signal quality and signal to background ratio. The primer pair "DMD 8ii F+R" amplifies a short sequence of 273 bp in exon 8. To our knowledge this is the first successful cyclic in

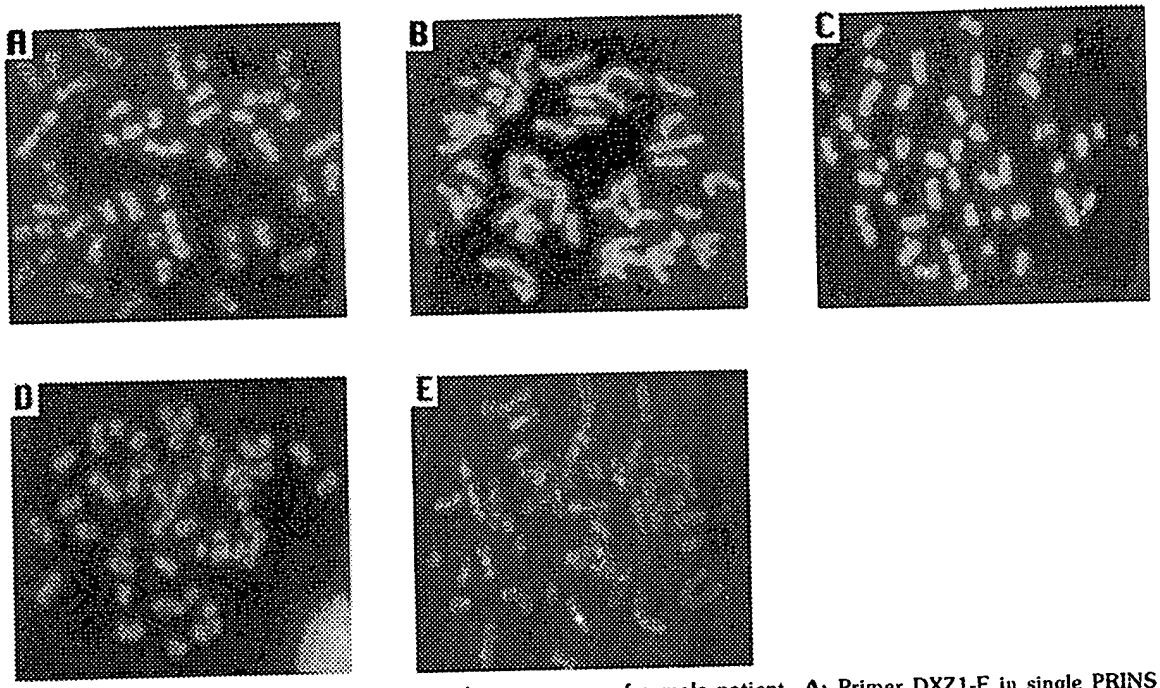

FIGURE 2. Labeling of DXZ1 on metaphase chromosomens of a male patient. **A:** Primer DXZ1-F in single PRINS reaction; **B:** Primer DXZ1-R in single PRINS reaction; **C:** Primerpair DXZ1-F+R in single PRINS reaction: revealing stronger signals than each primer alone; **D:** Primer DXZ1-F after 20 cycles of PRINS: no signal is obtainable; **E:** DXZ1-F+R after 20 cycles of PRINS: very strong signals. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

situ amplification of a single copy sequence on human metaphase chromosomes.

DISCUSSION

The main difficulty for every application of cyclic PRINS or in situ PCR-methods has been the enormous pressure shifts inside the closed reaction chambers due to the temperature changes during the cycling process. Commercially available reaction chambers and conventional slide sealing techniques with rubber gum [Gosden and Hanratty, 1993] or nail polisher

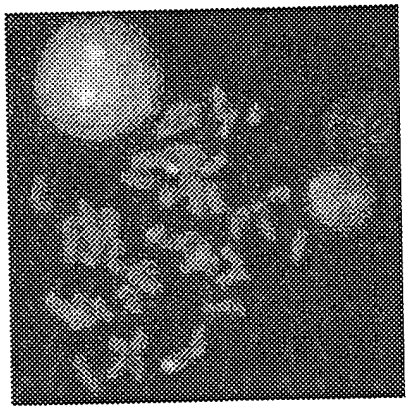

FIGURE 3. Primer D1Z2 after 20 cycles of PRINS: strong labeling of 1p36. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

FIGURE 4. Telomere primer $(CCCTAA)_7$ after 20 cycles of PRINS: labeling of all telomeres. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

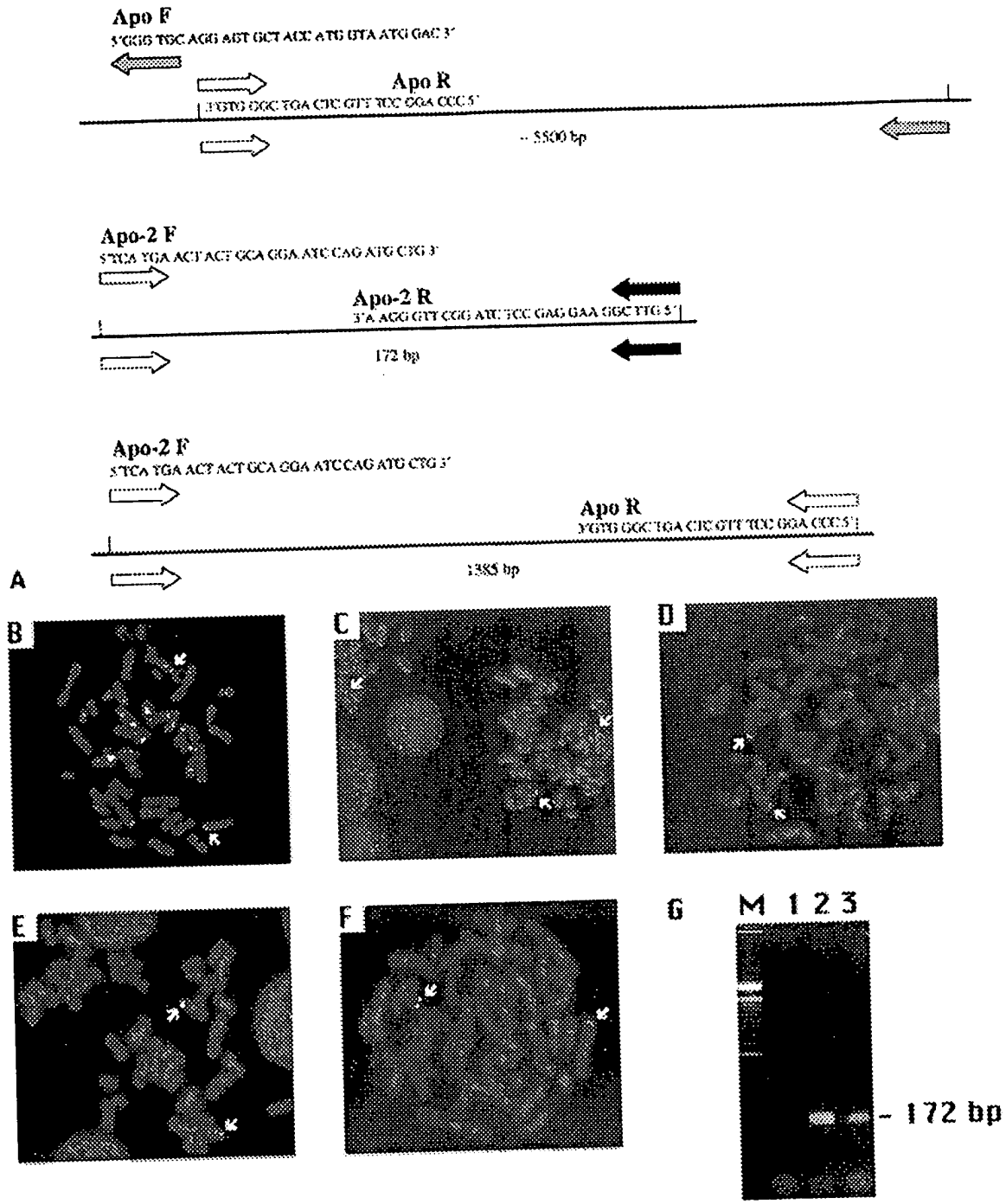

FIGURE 5. Apo(a) gene with low-copy repeats of Kringle IV motif on 6q26-27. **A:** Spacing of Primers Apo F, Apo R, Apo-2 F, Apo-2 R; **B:** Example for single PRINS (primers Apo-2 F + Apo R): weak signals; **C:** Primers Apo F + Apo R after 12 cycles of PRINS: strong signals; **D:** Primerpair Apo-2 F + Apo-2 R after 12 cycles of PRINS: strong signals; **E:** Primerpair Apo-2 F + Apo R after 12 cycles of PRINS: strong signals; **F:** Primerpair Apo-2 F + Apo-2 R after PCR-PRINS: strong signals (12 cycles with unlabeled nucleotides and three cycles with labeled nucleotides); **G:** Gel electrophoresis to control the cyclic PRINS reaction. (1): reaction mix before PRINS (negative control) / (2; 3): reaction mix after 20 cycles on the slide with different primer concentrations / (M): Marker.

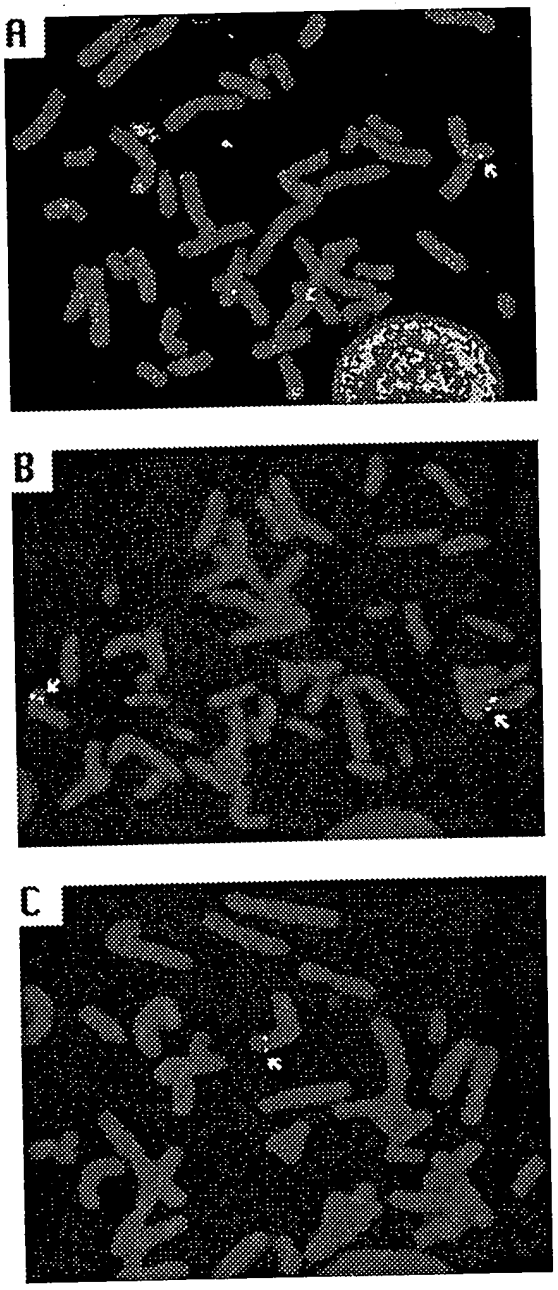

FIGURE 6. DMD gene on Xp21. **A:** Primerpair 8iiF+R after 12 cyles PRINS (male patient); **B:** Primerpair 8iiF+R after PCR-PRINS in a female patient (15 cycles with unlabeled nucleotides and five cycles with labeled nucleotides); **C:** Primerpair 8iiF+R after PCR-PRINS in a male patient (15 cycles with unlabeled nucleotides and five cycles with labeled nucleotides).

[Troyer et al., 1994] are not sufficiently effective according to our experiences. If the closed reaction chambers on slides can not provide perfect evaporation control over the entire reaction time, then concentration shifts are likely to compromise the result or at least limit the sensitivity of the method. By the use of open reaction chambers we have eliminated the problem of pressure shifts for in situ PCR and cyclic PRINS methods, thus allowing the amplification of their target sequences analogous to the tube PCR. The maintenance of constant reaction conditions through the entire protocol results in standardization and reproducibility of PRINS protocols. Constant stringency diminishes background staining and mismatch annealing. The open reaction chamber has also the advantage of providing access to the reagent mixture at any time. This allows both "hot starts" analogous to the tube PCR and "two step reactions" like PCR-PRINS [Hindkjaer et al., 1996] or nested PCRs.

Two-step in situ reactions can be carried out with our protocol in one cycler-run. We examined the reaction conditions for paired primers in our cyclic PRINS protocol by application of specific amplicons into the reaction-mix. The respective amplicons had previously been obtained on genomic DNA. In the reaction mix they served as easily accessible target equivalents. Gelelectrophoresis subsequent to the cycling reaction proved exponential amplification of the amplicons (Fig. 5G). We could furthermore demonstrate that higher concentrations of primers and labeled nucleotides can be used in our protocol and result in signal improvement without increase of background noise. With increasing numbers of reaction cycles, the chromosome morphology is suffering considerably like in any cyclic in situ application. We found the amount of 12 reaction cycles to yield the best results. Another finding is that chemical denaturation achieves a stronger signal intensity if the thermal denaturation is omitted for the first cycle of every cycling protocol.

Application of Cycling PRINS to Highly Repeated Sequences

From our results with the primer pair DXZ1-F and DXZ1-R, single primer "1p36" (D1Z2- se-

quence) and telomeric primer $(CCCTAA)_7$ we conclude that competitive forces exist in every cycling PRINS-reaction. And we assume that the balance of these forces is a primer-specific property which might reflect both the sequence and the local chromatin organization of the target sequence. In those cycling protocols in which DXZ1 primers were singly applied no signal was obtained: The diffusion of the product away from the target sequence through the repeated heating is stronger than the linear product amplification with the single primer. However, paired DXZ1 primers yielded strong signals in cyclic PRINS: Exponential product amplification is stronger than the diffusion of the labeled product away from the target. A different situation exists for the amplification with the single primer "1p36". Cyclic amplification resulted in very strong signals (Fig. 3), stronger than the signals obtained through a single PRINS-reaction: Linear amplification of the target sequence with this single primer exceeds product diffusion strongly. This is also the case for the telomeric primer $(CCCTAA)_7$.

Application of Our Cycling PRINS Protocol for the Detection of Low Copy Sequences

Exponential amplification is the case when the newly synthesized labeled product serves as target sequence in subsequent amplification cycles. That this is possible also under the restricting steric conditions of chromosomal DNA had been demonstrated by Therkelsen et al. [1993] with the exponential in situ amplification of the 171bp repeat in α-satellite DNA. For our primer-pair "Apo-2 F + Apo-2 R" we had also expected exponential in situ amplification but in cyclic PRINS it did not produce stronger signal intensities than amplification with single primers. This lead us to the question why a target sequence of 171bp within a highly repetitive DNA sequence is amplified exponentially while a target of 172bp within a low repeated sequence is amplified only in a linear mode. Our conclusion is that the amplification of an entire target sequence is not very likely to happen within the condensed chromatin.

In highly repeated sequences with thousands of possible binding sites, at least a few complete amplicons are produced, thus allowing the start of exponential amplification. The increase of signal intensity in linear amplification is most likely caused by partial amplification of the target sequence. While the incorporation of labeled nucleotides is no hindrance in the amplification of genomic DNA in tube PCR [Weier et al., 1991], there is some evidence that the label-nucleotide incorporation into chromosomal DNA does reduce the length of the obtained extension reaction and therefore the sensitivity of the method: Gosden and Hanratty [1991] compared the sensitivity of PRINS-reactions obtained with label-nucleotides of different sizes and found that nucleotides with a shorter C-linker result in higher sensitivity. This is in contrast to observations in other applications where longer C-linkers result in higher sensitivity. In the PRINS-reaction represents the bigger C-linker probably the bigger obstacle for the label incorporation into the target. In order to eliminate this eventual cause of steric hindrance from our reaction conditions we applied a two step cycling protocol with 15 cycles of unlabeled nucleotides, followed by 5 cycles with labeled nucleotides (Fig. 1). With this protocol we received the best results for all apo(a) gene primer pairs (Fig. 5F).

Application of Our Cycling PRINS Protocol for the Detection of Single Copy Sequences

Complex in situ methods like multiplex PCR-PRINS or nested PCR-PRINS, which theoretically should enable single copy gene detection, seem to be limited *through* the fact that chromosomal DNA does not show consistent properties in the same way as genomic DNA. The amplification of an entire target seems to be a rare event within the chromosomal DNA. Our experiments have shown that single copy gene in situ amplification is principally possible. However it seems to be the case that successful exponential amplification with the present methods is due to a rare primer-specific property and reflects the local chromatin organization of the target. In order to achieve the goal of routine single-copy gene detection it will be necessary to combine the cyclic PRINS method with a completely new technique that would provide better target access for the DNA polymerase prior to the PRINS reaction.

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REFERENCES

Abbs S, Yau SC, Clark S, Mathew CG, Bobrow M. 1991. A convenient multiplex PCR system for the detection of dystrophin gene deletions: a comparative analysis with cDNA hybridization shows mistypings by both methods. J Med Genet 28:304–311.

Beggs AH, Koenig M, Boyce FM, Kunkel LM. 1990. Detection of 98% of DMD/BMD gene deletions by PCR. Hum Genet 86:45–48.

Chamberlain JS, Gibbs RA, Ranier JE, Nguyen PH, Cascey CT. 1988. Deletion screening of the Duchenne muscular dystrophy locus via multiplex DNA amplification. Nucleic Acids Res 16:11141–11156.

Chamberlain JS, Gibbs RA, Ranier JE, Nguyen PH, Cascey CT. 1989. Multiplex PCR for the diagnosis of Duchenne muscular dystrophy. In: Innis M, Gelfand D, Sninski J, White T, editors. PCR protocols: a guide to methods and applications. Vol. 89. Orlando: Academic Press. p 272–281.

Gosden J, Hanratty D. 1991. Comparison of sensitivity of three haptens in the PRINS (oligonucleotide primed in situ DNA synthesis) reaction. Technique—a journal of methods in cell and molecular biology 3(4):159–165.

Gosden J, Hanratty D. 1993. PCR in situ: a rapid alternative to in situ hybridization for mapping short, low copy number sequences without isotopes. Bio Techniques 15(1):78–80.

Gosden J, Lawson D. 1994. Rapid chromosome identification by oligonucleotide-primed in situ DNA synthesis (PRINS). Hum Molec Genet 3(6):931–936.

Hindkjaer J, Terkelsen C, Koelvraa S, Koch J, Bolund L. 1996. Detection of nucleic acids (DNA and RNA) in situ by single and cyclic primed in situ labelling (PRINS): two alternatives to traditional in situ hybridization methods. In: Clark M, editor. In situ hybridization. Weinheim: Chapman & Hall. p 45–66.

Koch JE, Koelvraa S, Petersen ILF, Gregersen N, Bolund L. 1989. Oligonucleotide priming methods for the chromosome-specific labelling of alpha satellite DNA in situ. Chromosoma 98:259–265.

Koch J, Hindkjaer J, Koelvraa S, Bolund L. 1995. Construction of a panel of chromosome specific oligonucleotide probes (PRINS-primers) useful for the identification of individual human chromosomes in situ. Cell Genet Cytogenet 71:142–147.

Lackner C, Cohen JC, Hobbs HH. 1993. Molecular definition of the extreme size polymorphism in apolipoprotein (a). Hum Mol Genet 2(7):933–940.

Pellestor F, Girardet A, Andreo B, Charlieu JP. 1994. A polymorphic alpha satellite sequence specific for human chromosome 13 detected by oligonucleotide primed in situ labelling (PRINS). Hum Genet 94:346–348.

Pellestor F, Girardet A, Lefort G, Andreo B, Charlieu JP. 1995. Use of the primed in situ labelling (PRINS) technique for a rapid detection of chromosomes 13, 16, 18, 21, X and Y. Hum Genet 95:12–17.

Therkelsen AJ, Koch J, Koelvraa S, Hindkjaer J, Pedersen S, Bolund L. 1993. Repeated primed in situ labelling: formation and labelling of specific DNA-sequences in chromosomes and nuclei. Cytogenet Cell Genet 63:235–237.

Therkelsen AJ, Nielsen A, Koch J, Hindkjaer J, Koelvraa S. 1995. Staining of human telomeres with primed in situ labelling (PRINS). Cytogenet Cell Genet 68:115–118.

Troyer DL, Xie H, Goad DW, Skinner DZ. 1994. Use of a new technique to map the porcine α interferon gene to chromosome 1. Mammalian Genome 5:112–114.

Warburton PE, Greig GM, Haaf T, Willard HF. 1991. PCR amplification of chromosome-specific alpha satellite DNA: definition of centromeric STS markers and polymorphic analysis. Genomics 11:324–333.

Weier HUG, Lucas JN, Poggensee M, Segraves R, Pinkel D, Gray JW. 1991. Two-color hybridization with high complexity chromosome-specific probes and a degenerate alpha satellite probe DNA allows unambiguous discrimination between symmetrical and asymmetrical translocations. Chromosoma 100:371–376.